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13. ABSTRACT *(Maximum 200 words)*

RNA- or antibody-based strategies may provide novel strategies for inhibiting the expression of specific gene products that may be involved in prostate cancer cell growth and progression. Prostate cancer cells could escape hormonal control by constitutively expressing growth factors such as insulin-like growth factors (IGFs). This proposal is to test the hypothesis that IGF-II/IGF receptor signaling plays an important role in prostate cancer growth and progression. To prove the hypothesis, our newly constructed IGF-II ribozymes and single-chain antibodies against the IGF-I receptor (αIGFIR scFvs) are being used.

Specific Aim 1 is to test our hypothesis using already available PC-3 prostate cancer cell transfectants and control cells. Effects of intracellular expression of IGF-II ribozyme or αIGFIR scFv on cell growth, anticancer-drug induced apoptosis, and tumorigenesis will be investigated in cell culture and in athymic mice.

Specific Aim 2 is to take alternative approaches towards testing our hypothesis and developing novel gene and/or immunotherapy approaches for prostate cancer treatment.

RNA- or antibody-based strategies targeting growth factors in prostate cancer have never been evaluated. The proposed studies should thus lay the foundation for the development of novel strategies for prostate cancer treatment.

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Yoko Fujita Yang 9/20/99

PI Signature **Date**

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INTRODUCTION

Blockage of IGF-II/IGF-I receptor signaling by novel RNA- or antibody-based strategies should result in inhibition of prostate cancer cell growth and progression, and also induction of programmed cell death (apoptosis). The therapeutic potential of ribozymes and single-chain antibodies that target IGF-II and IGFIR, respectively, has never been tested. Now that the first sets of IGF-II ribozyme and anti-IGFIR single chain antibody (αIGFIR scFvs) have been designed, constructed, and expressed in a prostate cancer cell line in the PI's laboratory, it is possible to test their therapeutic potential for prostate cancer. Based on the first set of experiments, alternative strategies to improve the efficacy of ribozymes and αIGFIR scFvs are being explored.

BODY

Task 1. **To evaluate the effect of intracellular expression of IGF-II ribozyme and αIGFIR scFvs on cell growth, tumorigenesis, and apoptosis in cell culture and athymic mice (Months 1-24)**

- **To test the efficacy of the ribozyme expression on prostate cancer cell growth and apoptosis (Months 1-12)**

We have evaluated the effect of the IGF-II ribozyme expression on PC-3 cell growth, which demonstrated that PC-3 cells expressing the ribozyme do not grow but tend to die under the serum-free or low serum conditions while PC-3 cells expressing the inactive ribozyme or vector only grew or survive. This demonstrated that IGF-II ribozymes were able to lower endogenous IGF-II mRNA and protein levels, thereby blocking IGF-II/IGFIR signaling and inhibiting cell growth, which has been published (1)(Xu *et al.*, Endocrinology, **140**, 2134-2144, 1999; see Enclosure).

We have tried to evaluate whether PC-3 cells expressing the IGF-II ribozyme are prone to apoptosis. The experiments to measure apoptosis in general, however, did not work well in our laboratory. Thus, we stopped pursuing this research direction.

- **To test the efficacy of the αIGFIR scFvs expression on prostate cancer cell growth and apoptosis (Months 1-12)**

We lost the stable PC-3 clones expressing αIGFIR scFvs such as clones 19 and 26 shown in Fig. 3 of the original proposal. We attempted to isolate new stable PC-3 clones expressing αIGFIR scFvs, but failed to do so. Thus, we switched our strategy from stable transfection to transient transfection. The experiments are now underway.

- **To purify and characterize soluble αIGFIR scFv-Fc (Months 1-6)**

Purification and characterization of αIGFIR scFv-Fc are summarized below.

Purification of αIGFIR scFv or scFv-Fc: The cell culture supernatant of ~40 ml, collected from αIGFIR scFv-Fc-expressing transfectants, clone 1F12 or 1B8, was adjusted to pH 8.0 by adding 1/20 volume of 1.0 M Tris (pH 8.0), and passed through a Protein A-Sepharose CL 4B column (1 ml column). The column was washed with 10 column volumes of 100 mM Tris-HCl buffer, pH 8.0, 10 column volumes of 10 mM Tris-HCl pH 8.0. αIGFIR scFv-Fc was eluted from the column by 100 mM glycine buffer pH 3.0, and collected in 1.5 ml conical tubes containing 1/10 volume of 1M Tris (pH 8.0).

From culture media of 1F12 (40 ml) and 1B8 (38 ml), 1.8 and 3.2 mg of αIGFIR scFv-Fc, respectively, were purified. The level of αIGFIR scFv-Fc expression was thus calculated to be 45 and 85 μg/ml for 1F12 and 1B8, respectively. The purified αIGFIR scFv-Fc was used for biochemical and activity analyses of the engineered αIGFIR scFvs. To date, we have purified nearly 400 mg of the soluble αIGFIR scFv-Fc, which has been and will be used to characterize the effect of IGF signaling in cancer growth *in vitro* and *in vivo*.

Structural and functional analysis of αIGFIR scFv-Fc: SDS-PAGE analysis (7.5% gel) under reducing conditions revealed a single protein band of 53 kDa (Fig. 1A lanes 2 & 3), which showed immunoreactivity with anti-1H7 antibody (Fig. 1A lane 5). A single disulfide-linked protein of 120 kDa was detected by SDS-PAGE analysis (5% gel) under nonreducing conditions (Fig. 2B lane 1 & 2), indicating that αIGFIR scFv-Fc is a disulfide-linked dimer. Gel filtration showed that the dimer eluted at the expected position for the size of dimer, indicating that αIGFIR scFv-Fc does not form aggregates larger than a dimer in solution (data not shown).

First 15 N-terminal amino acids of the purified αIGFIR scFv-Fc were sequenced, which confirmed the authenticity of this recombinant antibody and the proper removal of the signal peptide. Binding constants of 1H7 and αIGFIR scFv-Fc for IGFIR determined by BIAcore were 1 x 10^9 and 1 x 10^8 M^{-1}, respectively.

Inhibition of IGF-I or IGF-II binding to the purified IGFIR by 1H7 or αIGFIR scFv-Fc: Effects of αIGFIR mAbs and αIGFIR scFv-Fc on IGF-I and IGF-II binding to the purified IGFIR are shown in Fig. 2. αIGFIR scFv-Fc inhibited IGF-II binding to the IGFIR more potently than IGF-I binding, which showed a similar trend to the previous observation with 1H7 (2). The inhibition potency by αIGFIR scFv-Fc was 10 times less than that by 1H7, which is consistent with their binding affinity difference. A control 2C8 mAb did not inhibit IGF-I or IGF-II binding to IGFIR.

Stimulation of autophosphorylation of the purified IGFIR by αIGFIR scFv-Fc: Autophosphorylation of the purified IGFIR was compared in the absence and presence of IGF-I, αIGFIR scFv-Fc, 1H7, and positive control poly L-lysine (PLL). Both antibodies similarly stimulated the β subunit phosphorylation (Fig. 4). Stimulation was the highest with PLL (12.8-fold) followed by IGF-I (3.8-fold), 1H7 (2.3-fold), and αIGFIR scFv-Fc (1.8-fold).

Effect of αIGFIR scFv-Fc on cell growth: The effect of extracellular addition of αIGFIR scFv-Fc or 1H7 on cell growth was determined by MTT methods using NIH3T3 cells overexpressing IGFIR. The average of four independent experiments is shown in Fig. 4. Cell growth was significantly inhibited after 4 days treatment with 10 or 100 nM 1H7 mAb (Fig. 4A) ($P<0.05$). In contrast, αIGFIR scFv-Fc was not as effective as 1H7 in inhibiting cell growth (Fig. 4B). After 4 days treatment with αIGFIR scFv-Fc, however, cell growth appeared to be inhibited by a dose-dependent manner. The highest dose used, 1000 nM αIGFIR scFv-Fc, was required to inhibit cell growth to the level similar to that achieved by 100 nM 1H7. This difference is presumably due to the difference in their affinity for the IGFIR.

- **To test the effect of the intracellular ribozyme expression on tumorigenesis and apoptosis in athymic mice (Months 6-24)**

Effects of intracellular ribozyme expression on tumorigenesis have been measured using PC-3 cells expressing ribozymes, control vector, and parental PC-3 cells in athymic nude mice. After the Experiment #1, we found that R4 and R6 lost the ribozyme expression while R39 was still expressing the ribozyme.

Experiment #1: Nude mice/PC-3 Rz clones (PC-3, vector (R6), vector (R4), R39, Rz)
Experiment #2: Nude mice/PC-3 Rz clones (PC-3, vector (R6), R39, Rz)
PC-3 tumor growth from above two experiments, #1 and #2 are shown in Figs. 5 and 6, respectively.

The results repeatedly indicated that Rz-expressing PC-3 clones, especially Rz, appear to grow *in vivo* more aggressively than control vector or parental PC-3 cells. These results are contrary to those of the *in vitro* cell growth experiments in which we showed that Rz-expressing PC-3 clones do not grow well as control PC-3 cells (1). In order to investigate this unexpected result we obtained, we are collaborating with Dr. Daisy De Leon, Loma Linda University.

- **To test the effect of the intracellular αIGFIR scFvs expression on tumorigenesis and apoptosis in athymic mice (Months 6-24)**

Since we were not able to obtain stable PC-3 transfectants expressing αIGFIR scFvs to evaluate the effect of the intracellular αIGFIR scFvs expression on tumorigenesis and apoptosis, we examined the effect of extracellular addition of αIGFIR scFv-Fc on PC-3 tumor growth in athymic mice. We did not find the significant effect of extracellular addition of αIGFIR scFv-Fc on PC-3 tumor growth in athymic mice (Fig. 7) while breast cancer MCF-7 tumor growth was significantly inhibited *in vivo* under the same conditions. We reason that this difference is due to the level of IGFIR expression which influence IGF dependency of the cancer cells.

Task 2. **To construct and evaluate alternative ribozymes and scFvs (Months 13-30)**

- **To construct and express alternative IGF-II ribozymes (Months 13-24)**

Rz constructs have been ligated into pLNL6MoMuLV; Rz/pLNL6MoMuLV (active Rz) and M/pLNL6MoMuLV (inactive mutant Rz). Retrovirus-mediated transfer to three prostate cancer cell lines, PC-3, DU145, LNCaP, is being carried out to compare the effect of Rz expression on prostate cancer cell growth.

- **To construct and express additional αIGFIR scFvs (Months 13-30)**

αIGFIR scFv constructs have been ligated into pHaNeoIRES; αIGFIR scFv/pHaNeoIRES (soluble form) and αIGFIR scFv-ER/pHaNeoIRES (ER-retained form). Retrovirus-mediated transfer to prostate cancer cell lines is being carried out to compare the effect of scFvs expression on prostate cancer cell growth.

- **To evaluate alternative IGF-II ribozymes in cell culture (Months 18-30)**

This part has not been started.

- **To evaluate alternative IGF-II ribozymes in cell culture (Months 18-30)**

This part has not been started.

DISCUSSION

Intracellular expression of an active IGF-II ribozyme suppressed endogenous IGF-II mRNA and protein levels, and thereby inhibited prostate cancer PC-3 cell growth. This result is consistent with our hypothesis that IGF-II plays a critical role in prostate cancer cell growth.

We have thus far failed to obtain stable PC-3 cell transfectants expressing αIGFIR scFvs. In addition, inhibition of IGF-II/IGFIR signaling by intracellular expression of IGF-II ribozyme reduced endogenous IGF-II levels only to ~40% of the control levels, which resulted in cell growth inhibition. The results not only confirmed our hypothesis, but also suggested that complete inhibition of the IGF-II/IGFIR signaling cannot be achieved since this signaling is absolutely required for PC-3 cell growth.

Thus, our focus for the Year 2 will be the retrovirus-mediated transient expression of IGF-II ribozyme and αIGFIR scFvs to three prostate cancer cell lines to test the effect of IGF-II ribozyme or αIGFIR scFvs expression on cell growth.

KEY RESEARCH ACCOMPLISHMENTS

1. The effect of intracellular expression of IGF-II ribozyme on prostate cancer PC-3 cell growth was evaluated. PC-3 cells expressing the ribozyme do not grow under the serum-free or low serum conditions while PC-3 cells expressing the inactive ribozyme or vector-transfected PC-3 cells grew. This result was consistent with our hypothesis that IGF-II plays a critical role in prostate cancer cell growth.

2. We have thus far failed to obtain stable PC-3 cell transfectants expressing αIGFIR scFv. In addition, inhibition of IGF-II/IGFIR signaling by intracellular expression of IGF-II ribozyme reduced endogenous IGF-II levels only to ~40% of the control levels.

   These "negative" results not only confirmed our hypothesis, but also suggested that complete inhibition of the IGF-II/IGFIR signaling cannot be achieved since this signaling is absolutely required for PC-3 cell growth.

REPORTABLE OUTCOMES

1. IGF-II ribozymes were able to lower endogenous IGF-II mRNA and protein levels, thereby blocking IGF-II/IGFIR signaling and inhibiting PC-3 cell growth, which has been published (Xu *et al.*, Endocrinology, **140**, 2134-2144, 1999; see Enclosure).

   The effect of intracellular expression of IGF-II ribozyme on prostate cancer PC-3 cell growth, which is an important outcome to demonstrate the role of IGF-II on prostate cancer growth, has been supported by this award.

   Stable PC-3 cell lines expressing IGF-II ribozyme as well as vector control cell lines have been developed.

CONCLUSIONS

Intracellular expression of an active IGF-II ribozyme suppressed endogenous IGF-II mRNA and protein levels, and thereby inhibited prostate cancer PC-3 cell growth. This result is consistent with our hypothesis that IGF-II plays a critical role in prostate cancer cell growth.

Inhibition of IGF-II/IGFIR signaling by intracellular expression of IGF-II ribozyme, however, reduced endogenous IGF-II levels only to ~40% of the control levels, which moderately reduced cell growth. This is reasonable since only PC-3 cell transfectants expressing IGF-II ribozyme, which are able to grow, can be achieved as stable clones.

We have been unable to obtain stable PC-3 cell transfectants expressing αIGFIR scFvs.

These "negative" results not only confirmed our hypothesis, but also suggested that complete inhibition of the IGF-II/IGFIR signaling cannot be achieved since this signaling is absolutely required for PC-3 cell growth.

Thus, our focus for the Year 2 will be the retrovirus-mediated transient expression of IGF-II ribozyme and αIGFIR scFvs to three prostate cancer cell lines to test the effect of IGF-II ribozyme or αIGFIR scFvs expression on cell growth.

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APPENDICES

- Figures 1 – 7
- Reprint of publication, *Endocrinology* **140**, 2134-2144

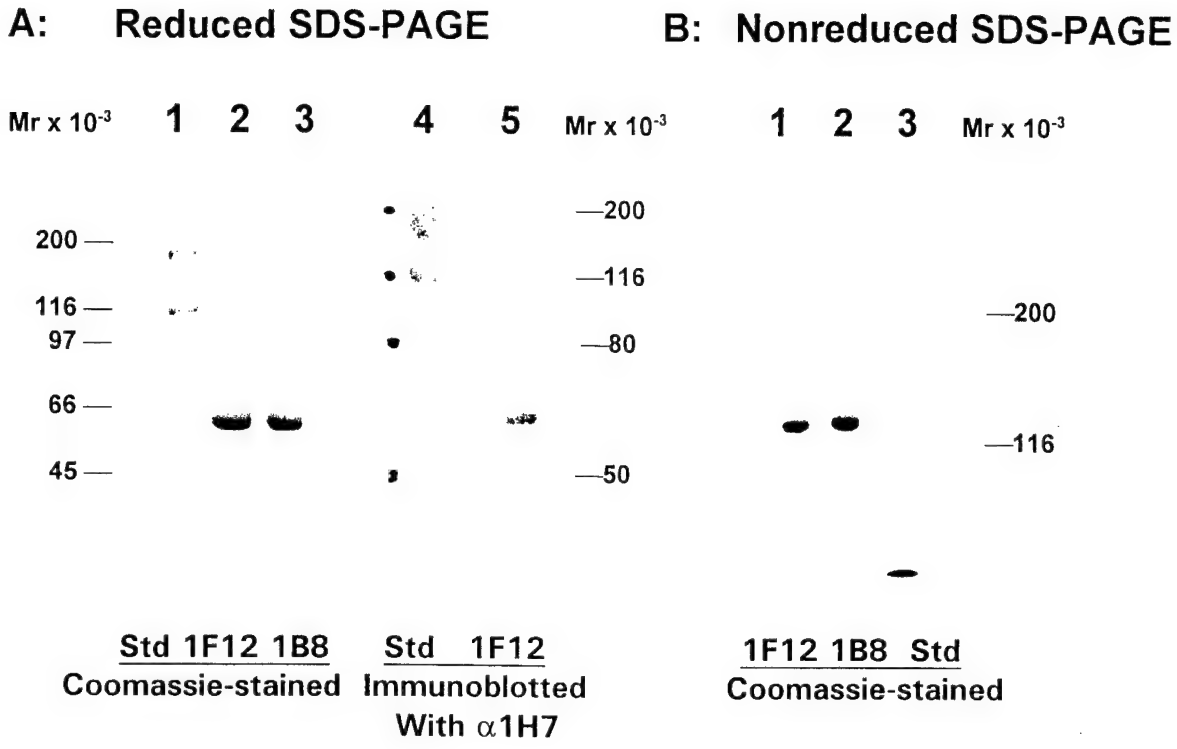

Figure 1. Purification of soluble form αIGFIR scFv-Fcs. A) SDS-PAGE under reducing conditions followed by Coomassie-staining (lanes 1-3) and immunoblotting (lanes 4 & 5) of the purified αIGFIR scFv-Fc. Shown are αIGFIR scFv-Fc purified from conditioned media of two independent stable clones, 1F12 and 1B8, as indicated. B) SDS-PAGE under nonreducing conditions, followed by Coomassie-staining of the purified αIGFIR scFv-Fc.

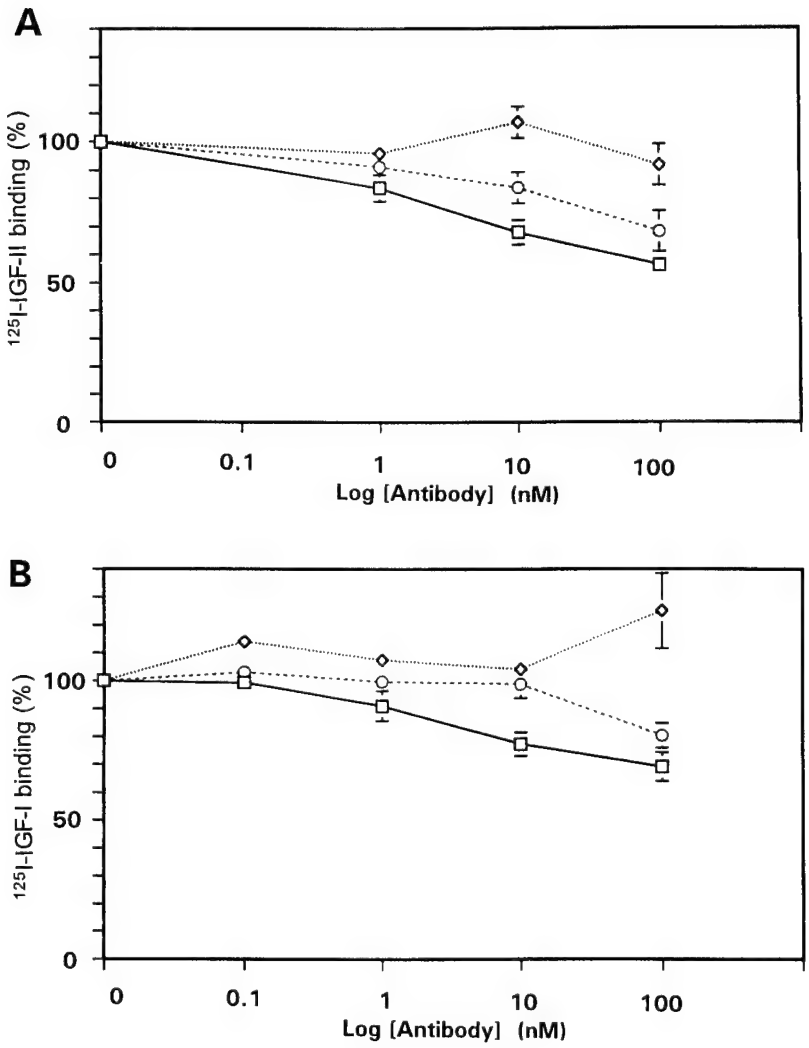

Figure 2. Effects of αIGFIR scFv-Fc and mAbs on ^{125}I-IGF-I (A) and ^{125}I-IGF-II (B) binding to the purified human IGF-I receptor. The binding activity is expressed as the per cent of binding in the absence of antibodies. Antibodies used are αIGFIR scFv-Fc (O), 1H7 (□), and control 2C8 (◊).

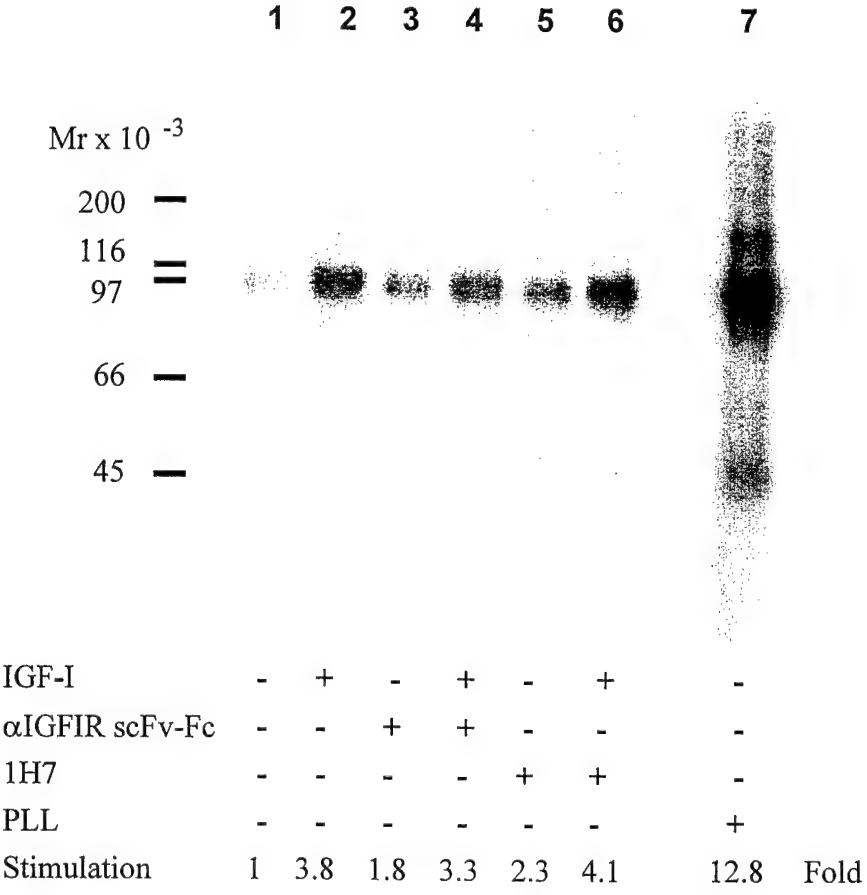

Figure 3. Effects of αIGFIR scFv-Fc and 1H7 on β subunit phosphorylation of the purified IGFIR. IGFIR was autophosphorylated in the presence of IGF-I and /or antibodies as indicated. Samples were analyzed by SDS-PAGE under reducing conditions. Shown is a phosphoimage of the gel. Fold-stimulation for the samples (lanes 2-7) was calculated by dividing the basal level phosphorylation (lane 1).

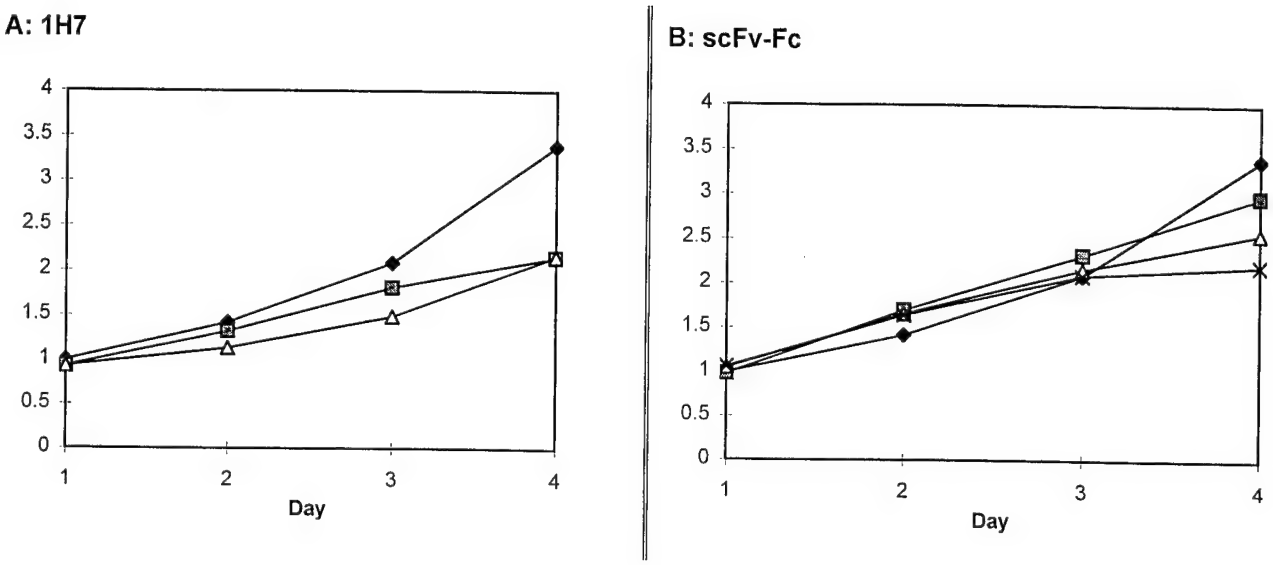

Figure 4. Effects of αIGFIR scFv-Fc and 1H7 on cell growth *in vitro*. NIH cells overexpressing IGFIR were cultured in DMEM supplemented with 2% FCS in the absence (♦) or presence of 10 nM (■), 100 nM (Δ), or 1000 nM (✕) of 1H7 (**A**) or αIGFIR scFv-Fc (**B**).

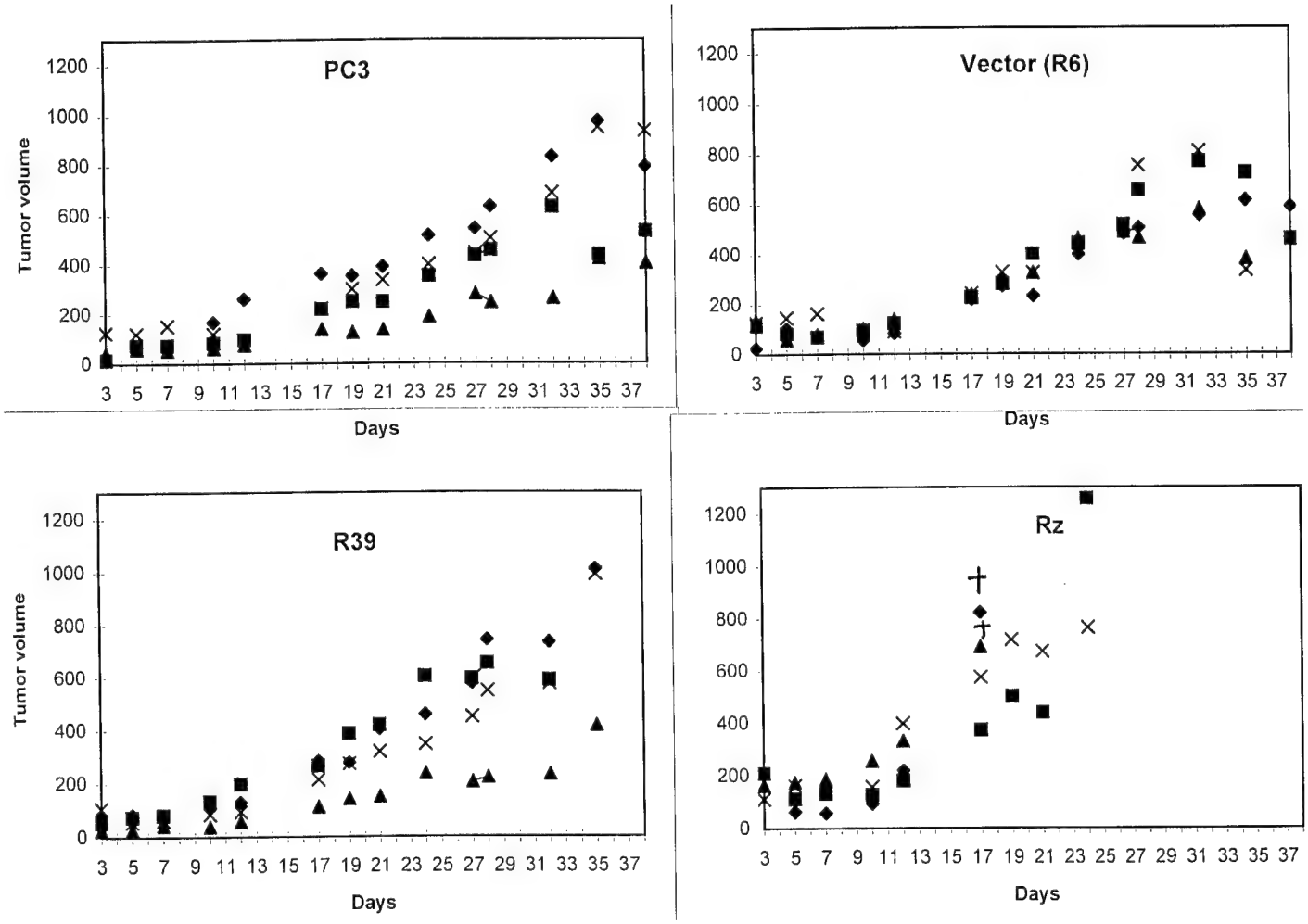

Figure 5. Effects of intracellular expression of IGF-II ribozyme on PC-3 tumor growth *in vivo* in nude mice. Nude mice, four each of 21 day-old mice, were inoculated s.c. in the flank with 10^7 PC-3 cells or PC-3 cells expressing Rz or vector control cells. The results of Experiment #1 are shown; PC-3 (parental), R6 (vector control), R4 (vector control; not shown), R39 (Rz expressing), and Vector (Rz expressing). Tumor growth was determined twice weekly by measuring length (l) and width (w), and the tumor volume was calculated as w^2 x $l/2$. Two mice indicated with (†) in Rz-group were sacrificed because tumors had been ulcerated.

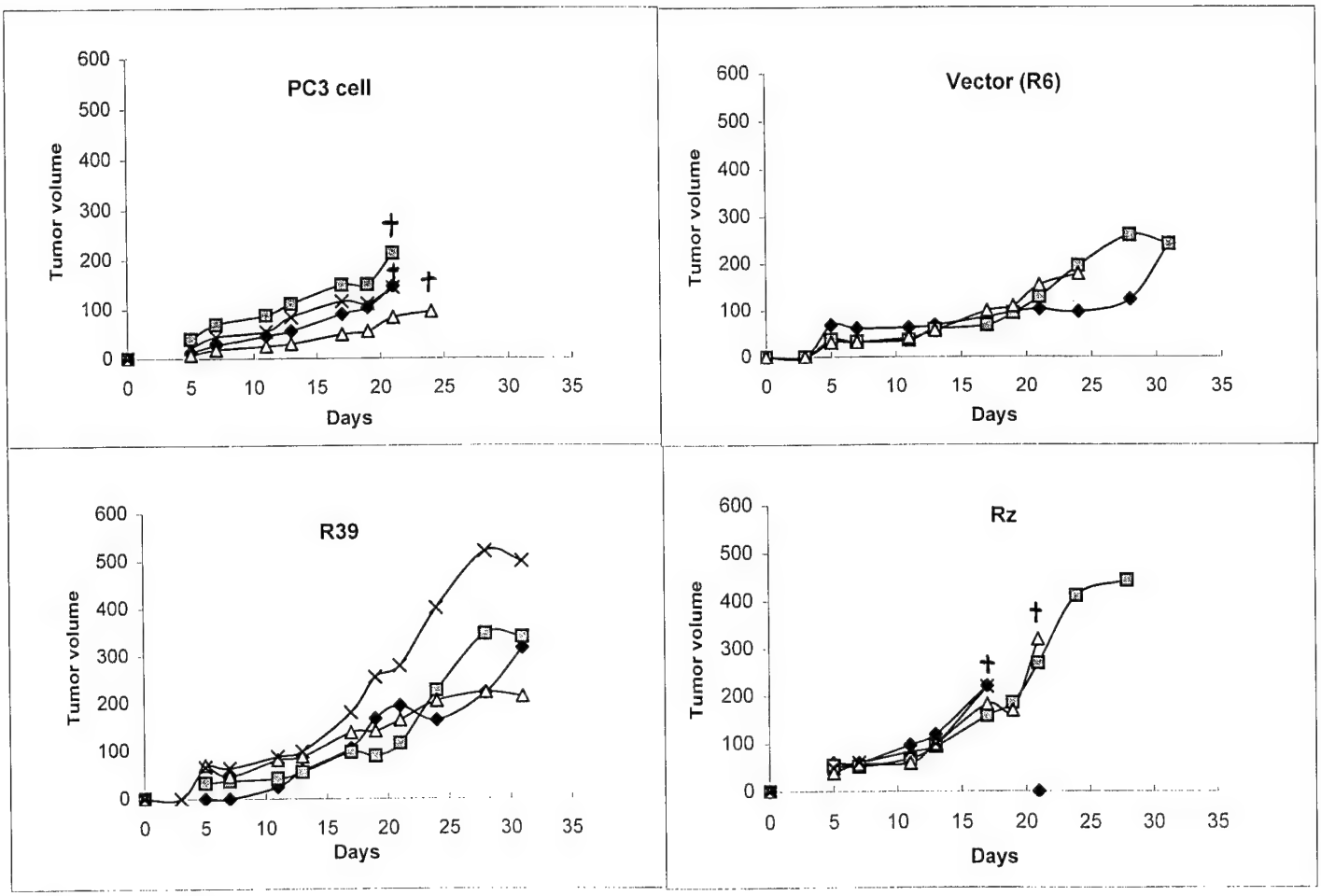

Figure 6. Effects of intracellular expression of IGF-II ribozyme on PC-3 tumor growth *in vivo* in nude mice. Nude mice, four each of 21 day-old mice, were inoculated s.c. in the flank with 10^7 PC-3 cells or PC-3 cells expressing Rz or vector control cells. The results of Experiment #2 are shown. PC-3, R6, R39, and Vector, correspond to parental cells, vector control, and two Rz-expressing cells, respectively. Four mice and three mice indicated with (†) in PC3 cell-group and Rz-group, respectively, were sacrificed because tumors had been ulcerated.

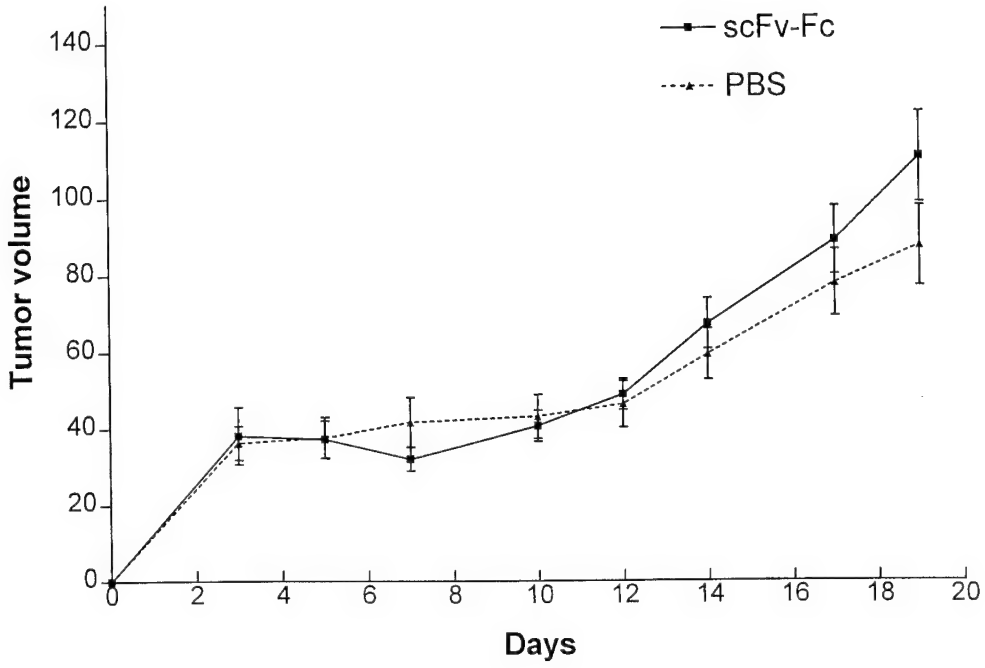

Figure 7. Effects of αIGFIR scFv-Fc administration on PC-3 tumor growth *in vivo* in nude mice. Three week old athymic mice were inoculated in the flank S.C. with 2 x 10^6 PC-3 cells on Day 0. On Day 5, αIGFIR scFv-Fc treatment started; intraperitoneal injections of αIGFIR scFv-Fc into 6 mice each (0.5 mg per mouse) twice weekly while control mice received PBS. Tumor growth was measured as described above.

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Hammerhead Ribozyme-Mediated Cleavage of the Human Insulin-Like Growth Factor-II Ribonucleic Acid *in Vitro* and in Prostate Cancer Cells*

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ABSTRACT

Insulin-like growth factor (IGF)-II plays an important role in fetal growth and development. IGFs are potent mitogens for a variety of cancer cells. A paracrine/autocrine role of IGF-II in the growth of breast and prostate cancer cells has been suggested. To test the role of IGF-II in cancer cell growth, hammerhead ribozymes targeted to human IGF-II RNA were constructed. Single (R)- and double (RR)-ribozymes were catalytically active *in vitro* whereas mutant ribozymes (M or MM) did not cleave IGF-II RNA. RR was more active than R. In human prostate cancer PC-3 cells, both R and RR similarly suppressed IGF-II messenger RNA (mRNA) levels (~40%) compared with the level in parental or M-expressing PC-3 cells. Polymerase II and III promoter-driven R similarly suppressed IGF-II mRNA levels. Suppression of IGF-II mRNA levels by R was associated with suppression of IGF-II protein levels. R- (or RR-) expressing PC-3 cells did not grow under serum-starved conditions and showed prolonged doubling times in the presence of 10% FCS compared with those of parental or M-expressing cells. These results substantiated that IGF-II plays a critical role in prostate cancer cell growth. (*Endocrinology* **140:** 2134–2144, 1999)

CANCER arises as a result of a series of molecular alterations in normal cells including up-regulation of growth factors that could facilitate uncontrolled cell growth. Insulin-like growth factor (IGF)-I and -II are important mitogens for a variety of cancer cells (1). The mitogenic actions of both IGF-I and -II are mediated via the IGF-I receptor (2–4). IGF-II also binds to the IGF-II/mannose-6 phosphate receptor and insulin receptor with high affinity while IGF-I binds to these receptors with low affinity. In addition, IGF-binding proteins bind IGFs with high affinity (5) and modulate IGF actions in both a positive and negative manner. Thus, the activity of IGFs in the local tissues depend, not only on the amount of IGFs produced, but also on the type and amount of IGF system components present.

IGF-II is a 7.5-kDa single-chain polypeptide of 67 amino acid residues, which is processed from its precursor (6). Previous studies suggested that an incompletely processed form of 15-kDa IGF-II is expressed more abundantly than the 7.5-kDa form in many cancers (1, 7–9). The 15-kDa form of human IGF-II was shown to have a mitogenic potency greater than that of 7.5 kDa (10). We showed that of 36 prostate, 17 breast, 10 bladder cancers, and 9 paraganglioma tissues examined, IGF-II was expressed in more than 50% of prostate, breast, and bladder tumors, and in 100% of paraganglioma tumors (9). Greater expression of the 15-kDa IGF-II relative to the 7.5-kDa IGF-II form was clearly demonstrated in all six prostate cancers and in one of the two breast and two of the four bladder cancers examined (9). The results are consistent with the hypothesis that the 15-kDa form of IGF-II expressed in cancerous cells contributes to autocrine cancer cell growth *in vivo*.

Evidence is accumulating that an enhanced IGF/IGF-I receptor (IGFIR) signaling leads to increased cancer cell proliferation and tumorigenesis as well as antiapoptotic effects (11, 12). For example, it has been demonstrated in experimental systems that overexpression of human IGFIR promotes ligand-induced neoplastic transformation (13) and tumorigenesis (14) in the presence of an active protein tyrosine kinase. An important role of IGFIR in mediating c-Myc-induced apoptosis of fibroblasts in low serum medium was first reported (15). Since then, a series of studies supporting the antiapoptotic role of IGFIR in cancer cells have been published (16–18). In brief, reduction of the number of IGFIR by introduction of antisense IGFIR cDNA caused extensive apoptosis *in vivo* in several transplantable human or rodent tumors (16).

In the case of prostate cancer, it was originally reported that IGF-I is responsible for autocrine growth of human prostate cancer cell lines including androgen-dependent LNCaP as well as hormone-independent DU145 and PC-3 cells (19). We and others more recently showed that IGF-II, but not IGF-I, is produced in those established human prostate cancer cell lines and suggested an autocrine regulation

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of DU145 and PC-3 cell growth by IGF-II (20–22). Furthermore, recent studies using *in situ* hybridization and immunohistochemistry indicated that epithelial cells rather than stromal cells in prostate tumors express IGF-II *in vivo* (9, 23). These data provided the basis for using prostate cancer as a model to test the hypothesis that cancer cell growth may be regulated by IGF-II in an autocrine manner.

Blockage of IGF-II/IGFIR signaling and subsequent effects on cell growth, transformation, and tumorigenicity have been reported. Examples of strategies to block IGF-II/IGFIR signaling include 1) inhibition of IGF-II expression by antisense oligonucleotides or RNAs (24–26); 2) inhibition of IGFIR expression by antisense oligonucleotides or RNAs (19, 27–28); 3) blockage of IGFIR by IGFIR monoclonal antibody such as αIR-3 (29, 30); and 4) blockage of IGF-mediated growth by IGF-binding proteins (20, 31). In addition, a number of anticancer drug agents have been shown to work, at least in part, by suppression of IGFIR action. For instance, suramin administration to breast, lung, and prostate cancer patients significantly reduced IGF-I and -II serum levels (32). Similarly, the antiestrogen tamoxifen has a powerful cytostatic effect in breast cancer cells, which in part is due to its inhibitory effect on the IGF-I/IGFIR axis (33).

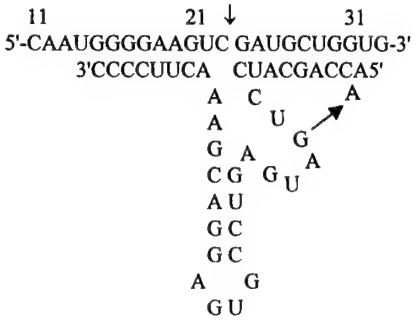
Ribozymes (RZs) are RNA enzymes that specifically cleave their respective target RNAs, thereby inhibiting the expression of specific gene products. Hammerhead-type RZs work in *cis* (intramolecularly) in nature, but separation of *cis*-acting RZs into two RNA fragments can convert them into *trans*-acting RNAs capable of site-specific cleavage of substrate RNAs. In the last 15 yr, RZs have progressed from an intriguing subject of scientific study to therapeutic agents for the potential treatment of both acquired and inherited diseases (34). It is becoming increasingly evident that RZs can serve the duel function of a tool to elucidate the functional roles of many gene products as well as therapeutic agents designed to functionally destroy deleterious RNAs. Suppression of IGF-II expression by IGF-II-specific RZs in cells has never been reported. We thus constructed catalytically active IGF-II RZs and expressed them intracellularly in human prostate cancer PC-3 cells.

Materials and Methods

Design and construction of IGF-II RZs

Two hammerhead RZs were designed and constructed to be complementary to the sequence near the translation initiation site of human prepro-IGF-II mRNA at nucleotides 16–30 and 16–46, respectively (35) (Fig. 1). As controls, activity of RZ was inactivated by introducing a point mutation of G to A in the catalytic core as shown in Fig. 1. Template DNAs for RZs and mutant RZs were prepared by filling in the opposite strands of two overlapping oligonucleotides by PCR. The oligonucleotides used for construction of the single ribozyme were 5′-ACGCGTCGACCAGCATCCT(A/G)ATGAGTCCGTGAGG-3′ (34 bases) and 5′-GCTCTAGAGCGGGGAAGTTTCGTCCTCACGGACTC-3′ (35 bases), which contained restriction enzyme *Sal*I and *Xba*I sites, respectively. The primers for the double RZs were 5′-ACGCGTCGACAGAAGGTCT(A/G)ATGAGTCCGTGAGGACGAAAGAAGCACCAGCAT-3′ (53 bases) and 5′GCTCTAGAGCGGGAAGTTT CGTCCTCACGGACTCAT(C/T)AGGATGCTGGTGCTT-3′ (52 bases), which contained restriction enzyme *Sal*I and *Xba*I sites, respectively. For both single and double RZs, the sequences that are complementary are *underlined*. Note that the position indicated as (/) represents equal ratios of both nucleotides incorporated to generate both active and inactive (mutant) RZ at the same time. The template DNAs for single and double RZ were prepared by five cycles of PCR

Single ribozyme



Double ribozyme

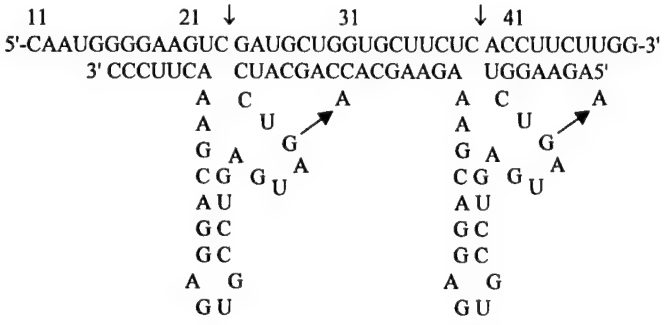


FIG. 1. Structure of the hammerhead RZ and target human IGF-II RNA. Secondary structures of single and double RZs and IGF-II substrate RNA are illustrated. Cleavage of the IGF-II substrate occurs at 3′ of the GUC or CUC as indicated with ↓. To produce an inactivated RZ, a highly conserved base, G5, in the catalytic domain, which is required for cleavage, was mutated to A as shown (G → A).

of 94 C for 1 min, 37 C for 1.5 min, and 72 C for 1.5 min, and five cycles of PCR of 94 C for 1 min, 33 C for 1.5 min, and 72 C for 0.5 min, respectively. After the PCR, the quality of PCR products was examined by PAGE. The PCR products were cleaned up, digested with *Sal*I and *Xba*I, and subcloned into the *Sal*I/*Xba*I sites of pTZU6 + 27 vector, which contained the human U6 promoter and pUC19 multiple cloning site (36). After the ligation reaction was carried out, the ligation products were electroporated into *Escherichia coli* XL-1 blue cells. The bacteria were plated on the LB agar containing ampicillin, X-gal, and isopropyl-β-D-thiogalactopyranoside. Plasmids were prepared from white colonies, and colonies containing the correct inserts were identified by *Sal*I/*Xba*I digestion and gel electrophoresis. To confirm authenticity of the RZs, plasmid DNAs were prepared and subjected to Southern blot analysis and DNA sequencing. Clones expressing single RZ (R) and its mutant (M, an inactive RZ in which a point mutation of G5 to A is introduced), and double RZ (RR), its mutant (MM), and double RZ with one mutant, RM or MR, were isolated. These plasmids, pTZU6+27/RZ, were used for both *in vitro* transcription of RZ as well as RNA polymerase (pol) III-driven expression of the RZ in mammalian cells.

Overlapping oligonucleotides that contained *Hin*dIII or *Xba*I sites were used to produce R or M, which was subcloned into *Hin*dIII/*Xba*I sites of pcDNA3 (pcDNA3/RZ). The primers used were; 5′-GCTCTAGAGCGGGGAAGTTTCGTCCTCACGGACTC-3′ and 5′-CCCAAGCTTGGGCAGCATCCT(A/G)ATGAGTCCGTGAGG-3′, in which complementary sequences are *underlined*. The template DNAs for R or M were prepared as described for pTZU6 + 27.

Subcloning of IGF-II substrate

To test the *in vitro* cleavage efficiencies of the RZs, a substrate human IGF-II RNA [−6 to +74, 80 nucleotides (nt)] was prepared by subcloning

a small fragment of IGF-II cDNA into pBluescriptII KS(+) (Stratagene, San Diego, CA). A DNA template for the IGF-II RNA was prepared by PCR from the full-length IGF-II cDNA (9). The primers used for the PCR were: 5′-CGGAATTCCGACACCAATGGGAATCCC-3′ and 5′-CGGGATCCCGGCAGGC AGCAATGCAGCACGA3-′, which contained a restriction enzyme site, *Bam*HI and *Eco*RI, respectively. PCR was performed as follows: 95 C for 3 min, and then 30-cycle amplification of 95 C for 1 min (denaturing), 55 C for 1 min (annealing), and 72 C for 1 min (extension).

The PCR product, digested with *Bam*HI and *Eco*RI, was ligated into pBluescript KS. Transformation of the ligation mixture was achieved by electroporation. Briefly, 3 μl of the ligation mixture were electroporated into *E. coli* XL1 blue cells. After LB was added, the bacteria were incubated at 37 C for 1 h to allow the antibiotic-gene express, and then plated on LB/1.5% agar containing 100 μg/ml Ampicillin, which had been layered with 100 μl of 0.05% X-gal and 75 mM IPTG, and grown at 37 C overnight. Plasmids were prepared from white colonies and their inserts were examined by *Bam*HI/*Eco*RI double digestion and gel electrophoresis. Colonies containing the right-size insert were selected. To confirm the authenticity of the IGF-II substrate sequence, plasmid DNAs were subjected to DNA sequencing.

Preparation of IGF-II mRNA substrate by in vitro transcription

The plasmids that contained the IGF-II substrate (−6 to +74) and the full-length IGF-II were linearized by *Bam*HI digestion and used as templates for *in vitro* transcription. Transcription reactions were carried out at 37 C for 1 h in 40 mM Tris-HCl buffer, pH 7.9, containing ~0.2 μg DNA template, 0.5 U/μl of T3 RNA pol, 20 mM $MgCl_2$, 10 mM NaCl, 10 mM dithiothreitol, 0.5 mM each of ATP, GTP, and uridine triphosphate, 0.05 mM cytidine triphosphate (CTP), 10 μCi of [α-^{32}P]CTP, and 1 U/μl of RNasin (Promega Corp., Madison, WI). After transcription, the RNAs were treated with RNase-free DNase I for 15 min and purified by electrophoresis in a 6% denaturing polyacrylamide gel for 1 h at 200 V. Before purification, a small aliquot of RNAs was removed to use for calculation of the specific activity. After electrophoresis, the gel was exposed to a Kodak XRP film (Eastman Kodak Co., Rochester, NY) for 1 min and the film was developed. The region of the gel containing the desired RNA band was excised and crushed into fine pieces. The IGF-II substrate RNA was eluted in an elution buffer overnight. The aqueous phase was removed and mixed with phenol-chloroform-isoamyl alcohol (25:24:1) to extract the RNA. The substrate RNA was precipitated with ethanol, redissolved in 20 μl diethyl pyrocarbonate-treated H_2O, and stored at −75 C.

In vitro transcription of RZs

The pTZU6+27/RZ plasmids were linerarized by *Xba*I digestion and used as templates for transcription of IGF-II RZ as described for substrate RNA preparation with two exceptions: only a trace amount of the radioisotope was used and T7 RNA pol was used instead of T3 RNA pol.

In vitro RZ cleavage assays

RZ assays were performed according to the methods previously described (37, 38). Briefly, RZs and substrate were heated independently for 1 min at 90 C in 10 μl of water. After cooling to 25 C, the reaction buffer was added to a final concentration of 10 mM $MgCl_2$, 140 mM KCl, and 50 mM Tris-HCl, pH 7.5. RZs and substrate were then combined and incubated at 37 C. The reaction was stopped by adding an equal volume of stop solution (0.5% of SDS/25 mM EDTA) and then 100 μl of phenol. The aqueous phase was brought to 100 μl. After vortexing, the aqueous phase was removed and the RNAs were precipitated with ethanol. The RNAs were analyzed by 6% polyacrylamide/8 M urea gel electrophoresis. Radioactive bands were visualized by autoradiography and quantitated by a PhosphorImager (Molecular Dynamics, Inc.).

The single turnover experiments using RZ excess over substrate were carried out to determine the first-order rate constant for cleavage of the substrate (39). The initial cleavage velocities under single-turnover conditions were determined at a constant substrate concentration of 1 nM and varying RZ concentrations. Reaction mixtures (10 μl) containing 0, 2.5, 5, 10, 20, 30, and 40 nM RZ and the substrate RNA (at a final concentration of 1 nM) were incubated at 37 C for 2 h. The reaction was stopped by the addition of 10 μl of the stop solution and analyzed by PAGE as described above. K_{cat}/K_m values were obtained by plotting the remaining fraction of the ^{32}P-labeled substrate RNA (Frac S) against the ribozyme concentration ([RE]) according to the following equation $k = -\ln(\text{FracS})/t = [RE] \times k_{cat}/K_m$ where k is the observed reaction rate and t is the reaction time of 2 h.

Cell culture and transfections

Human prostate cancer PC-3 cells were transfected with pTZU6+27/RZ or pcDNA3/RZ. The calcium-phosphate precipitation method was used to cotransfect PC-3 cells with pTZU6+27/RZ expression and neo vectors (40). Briefly, 24 h before transfection, exponentially growing cells were harvested by trypsinization and replated into 90-mm tissue culture dishes. Ten milliliters of RPMI 1640 medium with 10% FCS were added, and cells were incubated overnight at 37 C in a humidified incubator in an atmosphere to 5% CO_2. To those cells, were added 0.5 ml of 0.25 M $CaCl_2$ containing 18 μg of superhelical plasmid RZ DNA, 2 μg neo Vector DNA, and 0.5 ml of 2× PBS-buffered saline. The cells were incubated for 15 min at room temperature. RPMI 1640 medium with 10% FCS was added dropwise to the cells in the dishes, which were gently swirled. The cells were incubated for 16 h at 37 C in a humidified incubator in an atmosphere of 3% CO_2. The medium was removed by aspiration, and cells were rinsed twice with medium. Ten milliliters of fresh medium were added, and cells were incubated for 24 h at 37 C in a humidified incubator in an atmosphere of 5% CO_2. After 24 h incubation in nonselective medium to allow expression of the transferred genes to occur, the cells were trypsinized and replated in medium containing 600 μg/ml of G418. The medium was changed every 3 days for 4 weeks to remove the debris of dead cells and to allow colonies of G418-resistant cells to grow.

For the RNA pol II-driven RZ expression system, PC-3 cells were transfected by electroporation. Cells were grown to 70% confluence, trypsinized for 2 min, centrifuged in the table-top centrifuge, and resuspended in PBS buffer at a cell density of 5×10^6 cells per ml. This suspension was preincubated with 5–20 μg of DNA in 800 μl of PBS buffer on ice for 10 min with mixing, transferred to a cuvette, and immediately pulsed with the following settings: capacitance, 800 μFarads; resistance (R4), R4 (72 ohm); and charging voltage, 350 V. A 4-mm gap chamber was used in a BTX electroporator (San Diego, CA). After pulsing, the cells were left in ice for 10 min, transferred into the Petri dish, and cultured in the medium containing 600 μg/ml of G418 for at least 1 month.

Detection of IGF-II RZs expressed in PC-3 cells

RZ expression in G418-resistant clones was confirmed by RT-PCR. A set of primers for detection of RZ prepared were 5′-primer, 5′-TCGCTTCGGCAGCACGTCGAC-3′, containing a sequence corresponding to the junction between U6 promoter and the RZ, and 3′-primer, 5′-GGGAAGTTTCGTCCTCACGGA-3′, containing a sequence of the catalytic stem *underlined*. RT reaction was performed at 37 C for 45 min by mixing 4 μg of total RNA from the PC-3 transformants with 10 pmol of the 3′-primer in 50 mM Tris-HCl, pH 7.5, containing 10 mM dithiothreitol, 75 mM KCl, 3 mM $MgCl_2$, 1 mM of each deoxynucleoside triphospate, 800 U of Moloney murine leukemia virus reverse transcriptase, and 20 U of RNasin. RT reaction products, equivalent to 1 μg of total RNA from each clone, were processed through 30 cycles of PCR with denaturation at 94 C for 1 min, annealing at 47 C for 2 min, and synthesis at 72 C for 3 min, in a final volume of 20 μl. One half of the reaction product was analyzed in 2% agarose gel, stained with EtBr, and blotted onto a nylon membrane. The blot was hybridized with a specific ^{32}P-labeled oligonucleotide probe, 5′-TGGTC GTAGGACTACTCA-3′ (*underline and bold* indicate the complementary sequence to the catalytic stem and the position mutated in M, respectively) at 54 C for 16 h in 6× SSC, 5× Denhardt's solution, 1% SDS, and 100 μg/ml herring-sperm DNA. The membrane was washed three times in 2× SSC and 0.1% SDS for 30 min at 51 C. The bands hybridized with the ribozyme-specific probe were visualized by autoradiography. For detection of RZ in pcDNA3/RZ clones, PT-PCR was performed under the same conditions as above using 5′-primer, 5′-CCCACTGCTTACTGGCTTATCGA-3′, and 3′-primer, 5′-GGACAGTGGGAGTGGCACCTTC-3′.

Quantitation of IGF-II mRNA levels in PC-3 cells by quantitative competitive (QC)-PCR

The 5′- and 3′-primers used to detect RZ-mediated cleavage of IGF-II RNA in cells were 5′-CCAGCACCAATGGGAAT CCCAATGGGGAAG-3′ (−10 to +21) and 5′-GTATCTGGGGAAGTTGTCCGGAAGCAC GGTC-3′ (+294 to +324), respectively. pBluescript KS/IGF2, pBluescriptII KS(+) (Stratagene, La Jolla, CA) containing approximately 1 kb *Eco*RI fragment encoding the human precursor IGF-II (911–2067 nt) (9) was used to generate a new plasmid that encodes a competitor IGF-II sequence. Detailed methods will be published elsewhere (Xu, Z.-D., R. Mineo, S.-J. Liang, and Y. Fujita-Yamaguchi, manuscript in preparation). Briefly, a 110-bp *Sal*I/*Sal*I fragment was inserted into the IGF-II sequence between the PCR primers so that when the competitor IGF-II RNA was transcribed, it would provide a template for a PCR product that is 110 bp longer than that derived from the authentic IGF-II mRNA. In the presence of the competitor RNA, a 444- bp band, which was therefore produced in addition to the 334-bp band derived from an endogenous IGF-II RNA.

Conditioned medium

PC-3 cells and transfectants were seeded in 25-cm^2 flasks in the regular medium. The next day, cells were washed with PBS three times and grown for 2 days in 4 ml of RPMI 1640 containing 0.1% BSA. Condition media were collected and analyzed for IGF-II and -I as previously described (22). At the time of conditioned medium collection, cell numbers were determined with a hemocytometer, and the IGF-II protein level was expressed as nanograms/ml/10^5 cells.

Determination of cell proliferation

Cell growth was determined by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, which measures mitochondrial dehydrogenase activity that is active only in living cells. Cells (10^4 cells per well) were plated in 96-well plates containing 100 μl of medium and cultured 37 C for 4 days. At days 1, 2, 3, and 4, 20 μl of 0.5% MTT were added to the wells in triplicate. After incubation at 37 C for 4 h, the medium was removed and 100 μl of isopropyl alcohol supplemented with 0.05 N HCl were added. The color developed was quantitated by measuring absorbance at 540 nm.

Doubling times of parental PC-3 cells and transfectants were determined by time-lapse microscopy. Approximately 10^5 cells of each clone were seeded in medium supplemented by 10% FCS in a T-25 flasks and incubated at 37 C overnight. The flasks were placed under a phase contrast microscope (Nikon, Melville, NY) in a warm room. A good view of isolated cells was selected and video-recorded for 4 days. Doubling time for each clone was averaged from those of five to eight cell divisions.

Results

In vitro RZ cleavage reactions

Catalytic activity of the RZs constructed was examined using the shorter IGF-II RNA (−6 to +74) as a substrate unless otherwise stated.

Time course. The substrate, 7.7 nM, was incubated with 12.3 nM RZ, R or M, at 37 C for 10 to 210 min (substrate-RZ ratio of 0.6:1). As shown in Fig. 2, only the active RZ cleaved the substrate in a time-dependent manner. After incubation for 210 min, all the substrate was apparently digested by R.

Substrate-RZ ratio. RZ cleavage reactions were carried out under different substrate-RZ ratio conditions. After incubation at 37 C for 210 min, more than 80% of the substrate was cleaved by single RZ, R, at the substrate-RZ ratio of 1.2 or 0.6. In contrast, percent cleavage was markedly reduced at the substrate-excess condition.

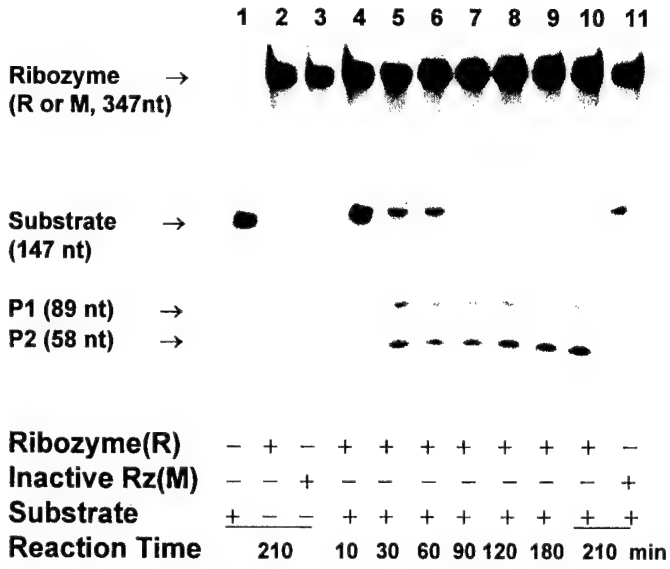


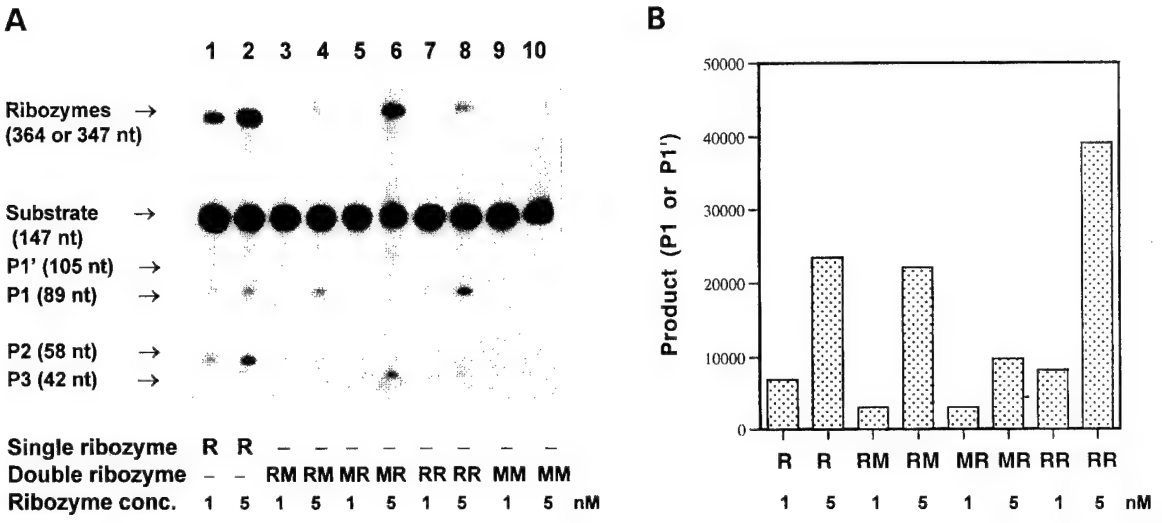
FIG. 2. Time course of RZ cleavage reaction by single RZs *in vitro*. Both RZs and substrate were ^{32}P labeled. A substrate-RZ ratio of 0.6:1 was incubated at 37 C for indicated time, 0–210 min. After cleavage reactions, RZ, substrate, and products were separated by 6% polyacrylamide/8 M urea gel electrophoresis. Nucleotide (nt) sizes of ribozymes, substrate, and products contain additional 5′-sequences derived from the vectors (see Figs. 1 and 3C for schematic illustrations). Note that the free substrate concentration after incubation for 3 h with M (lane 11) seems to become lower than the substrate only (lane 1), probably due to formation of a stable substrate/M RZ complex, which is resistant to denaturation conditions used in these studies.

In vitro cleavage reactions of single and double RZs. RZ activities of single RZ (R), double RZ (RR), its mutant (MM), and double RZs with one mutant (RM and MR) were analyzed. Figure 3A demonstrates that *in vitro* cleavage reactions of both single and double RZs produced the expected sizes of cleavage products as illustrated in Fig. 3C. Mutant double RZ (MM) did not cleave the substrate RNA at all. To compare relative cleavage efficiencies among various RZ constructs, the intensity of the product, either P1 or P1′, was quantitated. Figure 3B clearly indicates that R and RM are equally active whereas MR is less effective in cleaving the substrate IGF-II RNA than RM or R. The double RZ, RR, appeared the most active RZ of the four RZs that we constructed to target IGF-II RNA.

Comparison of RZ cleavage reactions by single and double RZs. Since the results of *in vitro* cleavage reactions suggested that RR is more active than R (Fig. 3), catalytic efficiencies of single and double RZs were further compared.

Kinetic analysis for the two RZs, R and RR, were performed under single-turnover conditions using the short IGF-II RNA substrate. The results summarized in Fig. 4 revealed K_{cat}/K_m = 1546 and 4772 $M^{-1}s^{-1}$, respectively. This indicates that the double RZ is approximately 3-fold more efficient in cleaving the substrate IGF-II RNA than the single RZ *in vitro* ($P < 0.05$).

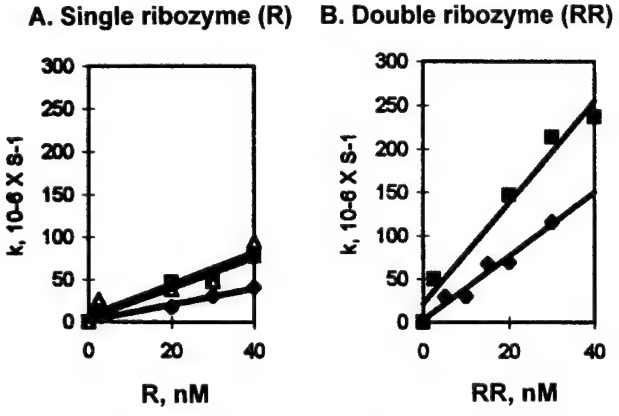
The catalytic efficiencies of single and double RZs were also examined using the precursor IGF-II RNA (1157 nt) as a substrate. Both RZs cleaved the IGF-II RNA substrate after incubation for 16 h (Fig. 5A, lanes 11 and 12). The time course



C

Ribozymes	Substrate ~~~~~~~~~~ −6 IGF-II RNA 76	Products (nt)
R	↑	89 + 58
M		147
RM	↑	89 + 58
MR	↑	105 + 42
RR	↑↑	89 + 16 + 42
MM		147

FIG. 3. Comparison of RZ cleavage reaction by single and double RZs *in vitro*. A, Autoradiogram. Cleavage reactions by single RZ (R), double RZ (RR), its mutant (MM), and double RZs with one mutant, RM or MR, were compared. Both RZs and substrate were ^{32}P labeled. Note that RZs were ^{32}P labeled with only trace amounts of radioactivity while the substrate was fully ^{32}P labeled. The differing intensities of RZs are due to differences in the levels of radioactivity incorporated during *in vitro* transcription of template encoding different RZ constructs. RZ reactions were carried out at 37 C for 210 min at a substrate concentration of 5 nM in the presence of 1 or 5 nM RZ (R, RM, MR, RR, or MM). After cleavage reactions, RZ, substrate, and products were separated by 6% polyacrylamide/8 M urea gel electrophoresis. Nucleotide (nt) sizes of the RZ, substrate, and products are indicated. B, Relative intensity of the RZ product, P1 or P1′. Cleavage efficiencies of the RZs shown in lanes 1–8 in panel A were determined by quantitating the intensity of the products, P1 or P1′. C, Schematic illustration of IGF-II RNA substrate and RZ products. The IGF-II substrate and RZ cleavage sites are presented schematically to show nt sizes of the products derived from each RZ or inactive RZ. An additional 5′-sequence which is attached to the substrate is shown as ~~~~~.



	Kcat/Km, M^{-1} s^{-1}	
	R	RR
Experiment 1 ■	1803	5840
Experiment 2 ◆	965	3704
Experiment 3 Δ	1870	
Mean ± SD*	1546 ± 411	4772 ± 1068

*P<0.05

FIG. 4. Comparison of single and double RZs *in vitro* by single-turnover experiments. Single turnover experiments with RZ in excess over substrate were carried out and K_{cat}/K_m values were calculated as described in *Materials and Methods*. In Exps 1 and 2, R and RR were assayed in parallel.

experiment, however, revealed that during the first 3 h of incubation, RR cleaved the substrate in a time-dependent manner whereas R did not appear significantly to cleave the substrate (Figs. 5A, lanes 1–8, and Fig. 5B). This result is consistent with that of the kinetic analysis that indicated that RR is more active than R *in vitro*.

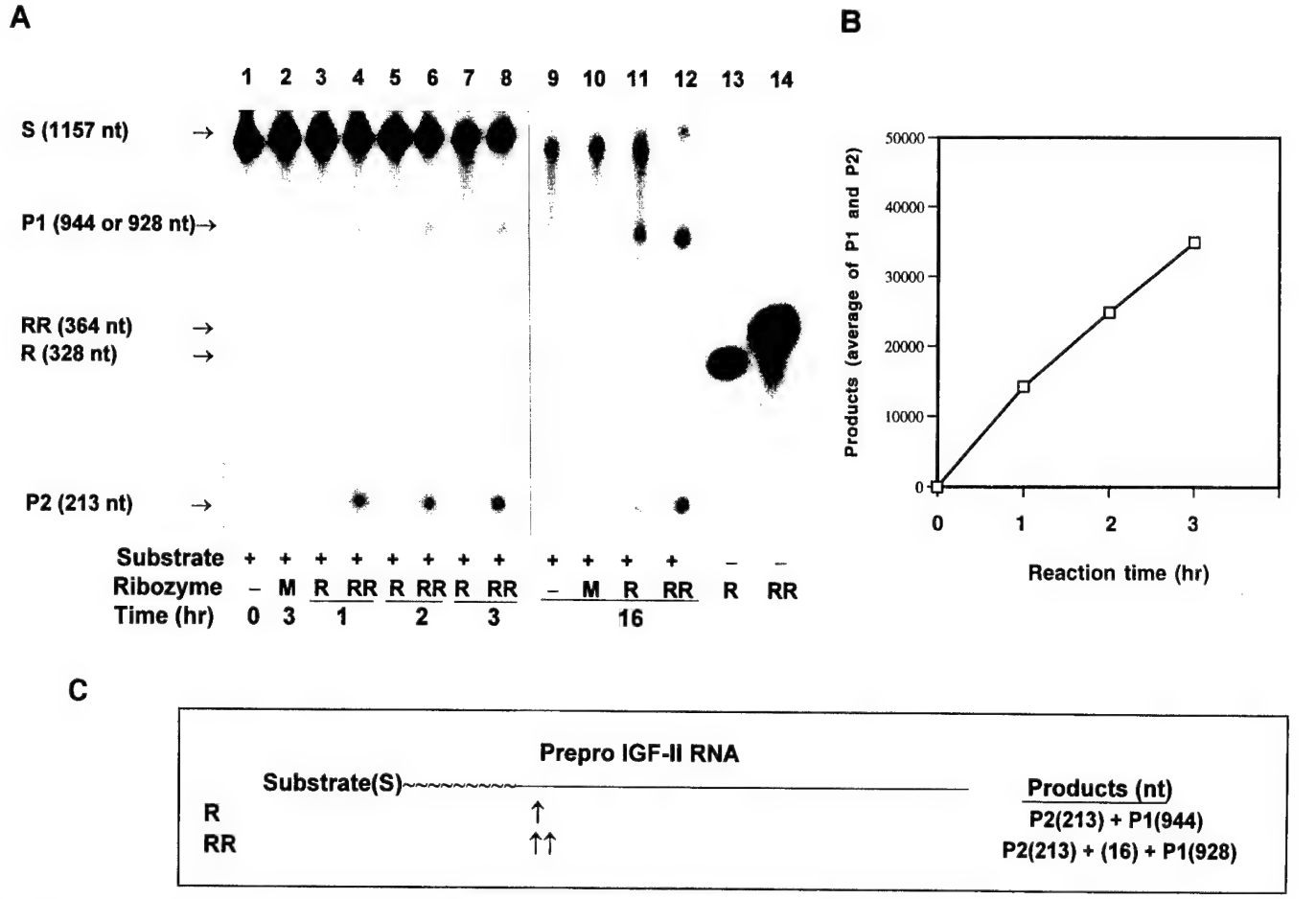


FIG. 5. RZ cleavage reaction by single and double RZs *in vitro* using the precursor IGF-II RNA as a substrate. A, Autoradiogram. Prepro-IGF-II RNA (1157 nt) was synthesized from BluescriptII KS encoding the prepro-IGF-II cDNA. Both RZs and substrate were ^{32}P labeled. RZ reactions were carried out 37 C for 1–16 h at a substrate concentration of 15 nM with 1.2 mM single or double RZ. The results of two independent experiments are shown; lanes 1–8 and lanes 9–14 are from Exp 1 and 2, respectively. B, Relative intensity of the RZ products. Cleavage reaction by RR was shown as average of P1 and P2 produced after incubation of 1, 2, and 3 h. C, Schematic illustration of IGF-II RNA substrate and RZ products. RZ cleavage sites and expected products are schematically presented.

Intracellular cleavage of IGF-II mRNA by RZs

Intracellular activity of the RZs was determined in PC-3 cells that were stably expressing RZs. Two expression systems that use RNA pol III and II were used in this study. While RNA pol II expression systems have been developed for years and widely used for the expression of proteins, RNA pol III expression systems have been suggested to be advantageous for stable, high steady-state expression of short chimeric RNAs since RNA pol III-driven chimeric transcripts are more abundant than poly(A)$^+$ RNAs in mammalian cells (41).

Stable expression of RZs in PC-3 cells driven by RNA Pol III promoter (pTZU6+27 vector).

Detection of RZ mRNA in PC-3 cell transfectants. Expression of R, M, and RR in four, two, and two transfectants, respectively, was confirmed by EtBr-stained agarose gel electrophoresis of RT-PCR products prepared from DNase I-treated RNA (data not shown). No PCR amplification was made when PCR was performed from the corresponding RNA preparations without RT (data not shown). RT-PCR products derived from single and double RZs migrated at the same positions as PCR controls for R and RR, respectively, prepared from the plasmids encoding R and RR (data not shown). The authenticity of R, M, and RR was confirmed by Southern blot analysis using an oligonucleotide probe complementary to the R sequence (data not shown). The mutant RZ did not hybridize with the R-specific probe at 54 C due to a one-base mismatch in the probe sequence.

Quantitation of IGF-II mRNA levels in PC-3 cell transfectants. IGF-II mRNA levels in parental and transfected PC-3 cells were determined by QC-PCR using a competitive IGF-II RNA as a control (Fig. 6A). Endogenous IGF-II mRNA levels in PC-3 cells expressing R or RR were suppressed by 62% or 68%, on average, compared with that of parental PC-3 cells, whereas cells expressing M had little effect (12% suppressed) on the IGF-II mRNA levels (Fig. 6, A and B) as determined by quantitative RT-PCR.

Effect of RZ expression on PC-3 cell growth. The effect of intracellular expression of RZs and the subsequent reduction of IGF-II mRNA and protein levels on cell growth was eval-

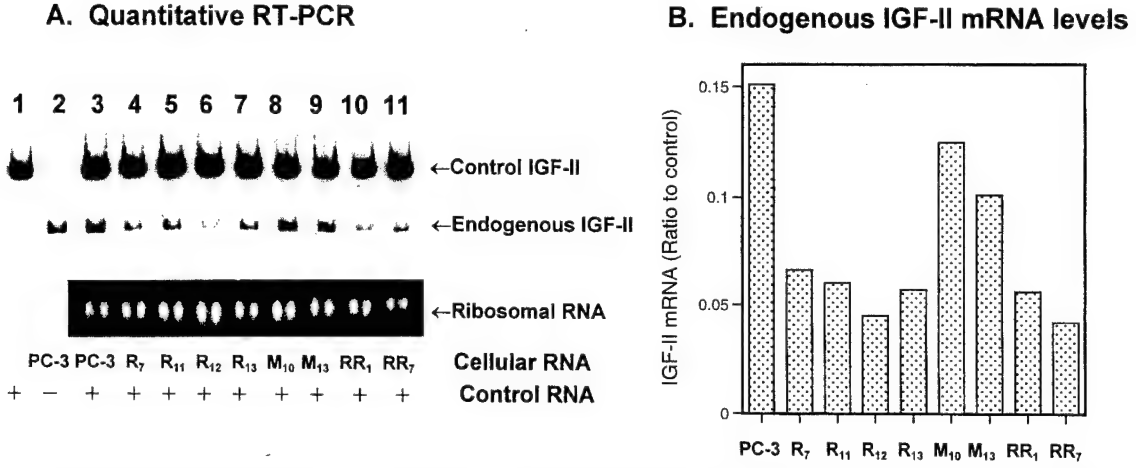


FIG. 6. PC-3 transfectants expressing single (R), double (RR) or inactive single (M) RZ under the control of Pol III promoter. A, Endogenous IGF-II mRNA levels as determined by QC-PCR. Quantitative RT-PCR was performed using IGF-II-specific primers and 1 μg of total RNA prepared from parental PC-3 cells (lane 3) and transfectants (lanes 4–11) in the presence of a competitor IGF-II RNA (110 bp longer than the authentic IGF-II mRNA). Lanes 1 and 2 show PCR products from control IGF-II and PC-3 RNA, respectively. B, Endogenous IGF-II mRNA levels in parental PC-3 cells and R-, RR-, or M-expressing PC-3 cells. Endogenous IGF-II mRNA was expressed as the ratio to control RNA, which is further normalized by 18S ribosomal levels shown in panel A.

TABLE 1. Effect of RZ expression on PC-3 cell growth

	Relative cell growth after 2 days in SFM (mean ± SE)[a]
Parental PC-3 cells	1.23 ± 0.02 (n = 2)
R-expressing PC-3 cells	0.58 ± 0.13 (n = 5)[b]
RR-expressing PC-3 cells	0.4 (n = 1)
M-expressing PC-3 cells	1.61 ± 0.33 (n = 2)

PC-3 cells and transfectants were plated in 24-well plates (1.3×10^4 cells per well), and cell numbers were counted after 2 day-culture in serum-free medium. Cell growth is expressed as a ratio of the cell number at day 2 to the original cell number seeded.

[a] The relative cell growth values are mean values of those of independently isolated stable clones. Numbers of clonally selected transfectants are indicated in *parentheses*. For parental PC-3 cells, n = 2 means two independent measurements of the cell line.

[b] Significantly different ($P < 0.01$) by Newman-Keuls post hoc test compared with parental PC-3 cells.

uated under serum-free conditions. Parental PC-3 and transfectants were grown in serum-free medium (SFM) for 2 days. While cell numbers of parental PC-3 cells or M-expressing cells increased, those of R- or RR-expressing cells decreased (Table 1), indicating that catalytically active RZ expression is inhibitory for PC-3 cell growth. These results suggest that cleavage of IGF-II mRNA by R or RR, but not the potential antisense effect of M *per se*, is required for inhibition of PC-3 cell growth.

Stable expression of RZs in PC-3 cells driven by RNA Pol II (pcDNA vector). While the transcripts expressed by the RNA pol III U6 + 27 system are nuclear (36), capped, polyadenylated RNA pol II transcripts are more likely to direct RZ transcripts to the same intracellular compartment as the targeted IGF-II mRNA in the cytoplasm. To compare RZ activity under the control of RNA pol II promoter, PC-3 cells were transfected with pcDNA3/RZ.

Detection of RZ mRNA in PC-3 cell transfectants. Of 10 transfectants examined, expression of R was confirmed in 4 independent clones by RT-PCR (Fig. 7A). EtBr-stained agarose gel electrophoresis of the RT-PCR product also identified six G418-resistant clones that do not express R. They were used as vector-control cells below.

Measurements of IGF-II mRNA and protein levels in PC-3 cell transfectants. IGF-II mRNA levels in parental and transfected cells were determined by QC-PCR (Fig. 7B). Endogenous IGF-II mRNA levels for parental, R-expressing, and vector-transfected PC-3 cells were 0.2 (n = 1), 0.045 ± 0.005 (n = 4), and 0.122 ± 0.018 (n = 6), respectively. Endogenous IGF-II mRNA levels in R-expressing PC-3 cells were thus reduced to approximately 40% level when compared with those of six transfectants that do not express R (vector-control), and to about 20% level when compared with that of parental PC-3 cells. In parallel to the IGF-II mRNA levels, IGF-II protein levels secreted into conditioned media by R-expressing PC-3 cells were significantly lower than those of parental or vector clones, while that of the vector-transfected cells was modestly reduced (Table 2)[1].

Effect of RZ expression on PC-3 cell growth as measured by the MTT method. The growth of parental PC-3 cells and the vector- or R-transfectants, shown in Fig. 7, were analyzed in SFM (Fig. 8A) or in the presence of 2% FCS (Fig. 8B). The results clearly demonstrate that PC-3 cells expressing the IGF-II RZ do not grow well under serum-free or 2% FCS conditions. The results are similar to the observation we had for R- or RR-expressing PC-3 cells under the control of RNA pol III promoter. Since the R-expressing PC-3 cells were isolated after culturing those transfectants in the medium containing 10% FCS, they must have been able to grow in the presence of 10% FCS (see time-lapse microscopy experiments below).

[1] IGF-I protein levels secreted into conditioned media by parental and PC-3 cell transfectants were determined. The IGF-I levels measured, however, were too low to be informative given that PC-3 cell IGF-I levels are below accurate levels of detection (22).

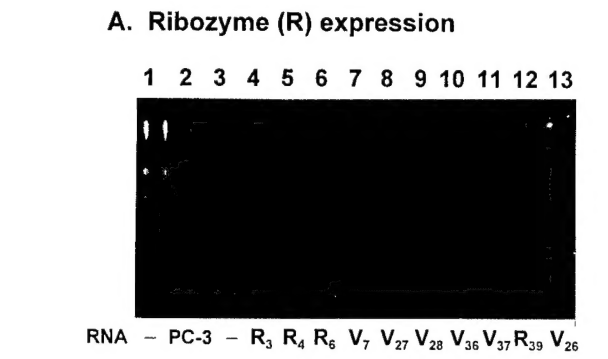


FIG. 7. Expression of RZ under the control of Pol II promoter. A, Detection of RZ expression by RT-PCR. Experiments were performed as described in Fig. 6A. B, Endogenous IGF-II mRNA levels in parental PC-3 cells, R-expressing PC-3 cells, and vector-controls as determined by QC-PCR. Experiments were performed as described in Fig. 6B. C, IGF-II protein levels in conditioned media. IGF-II protein levels in conditioned media harvested from parental cells and PC-3 cell transfectants were measured in duplicate.

Effect of RZ expression on PC-3 cell growth as measured by time-lapse microscopy. Analysis of cell division of parental PC-3 cells, vector-transfected cells (V37), and R-expressing PC-3 cells (R4, R6, and R39) revealed doubling times (mean ± SEM) of 28.3 ± 0.39 (n = 8), 32.3 ± 1.0 (n = 3), 37 ± 1.1 (n = 6), 38.6 ± 2.3 (n = 5), and 42.5 ± 2.8 (n = 2) h, respectively (Table 3). This result indicates that PC-3 cells expressing the IGF-II RZ showed a significantly prolonged doubling times compared with parental PC-3 cells, while the doubling times of vector-transfected PC-3 cells are not significantly different from those of the parental cells.

Discussion

Hammerhead RZs against human IGF-II RNA, which we have constructed, were catalytically active *in vitro*. The catalytic efficiencies, however, varied among differently designed IGF-II RZs. The results can be summarized as follows; RR > R ~ RM > MR. This indicates that the first RZ cleavage site may be a better target than the second RZ cleavage site since the cleavage of the former by R, RM, or RR was more

TABLE 2. IGF-II protein levels secreted into conditioned media

	Exp	IGF-II protein level (ng/ml/10^5 cells)
Parental PC-3 cells	1	6.22
	2	5.85
Vector-transfected PC-3 cells	1	4.07
	2	4.5
R-expressing PC-3 cells (R3)	1	2.07
	2	2.91
R-expressing PC-3 cells (R4)	1	2.95
	2	2.5
R-expressing PC-3 cells (R6)	1	2.31
	2	2.06
R-expressing PC-3 cells (R39)	1	1.07
	2	3.68

IGF-II protein levels in conditioned media harvested from parental PC-3 cells and transfectants were measured in duplicate. IGF-II protein levels in the pooled data of R-expressing PC-3 cells were significantly different ($P < 0.01$) by Newman-Keuls post hoc test compared with parental and vector-transfected PC-3 cells.

efficient than that of the later by MR. Of three double RZs examined, RR was more active than RM and MR. RR was also 3-fold more efficient in cleaving a substrate IGF-II RNA than R as judged by kinetic analysis under single-turnover conditions. Both RM and R were equally active despite the difference in the length of antisense arms, 29 and 17 nt, respectively. These results suggest that the tandem repeat of two catalytic domains enhances the catalytic efficiency of the RZ rather than the longer antisense arms. When a longer RNA substrate was used, RR was much more effective in cleaving the substrate than R. However, the cleavage of the longer substrate (1157 nt) required a much higher concentration of RR than that of the shorter substrate (147 nt). These observations are generally in good agreement with the results of previous studies that systematically analyzed the effects of catalytic core tandem repeats (42) or antisense arm lengths on RZ cleavage reactions *in vitro* (43).

Single and double RZs were stably expressed in human prostate cancer PC-3 cells under the control of RNA pol III or pol II promoter. Stable transfectants were isolated and used to determine the efficacy of the RZ activity in cells and effects of active RZs on cell growth. Establishment of quantitative assays for the target RNA was necessary for the detection of RZ cleavage reactions *in vivo* (in cell line). The QC-PCR method was used in this study because it is very sensitive, specific, and quantitative (44). In contrast to most of the RT-PCR methods that use two pairs of primers, the competitor IGF-II RNA shares the same sequences for primer binding as the target endogenous IGF-II mRNA. But, it contains an additional 110-base long insert that will give a PCR product larger than the PCR product generated from the target endogenous IGF-II mRNA. This 110-base difference in size was sufficient for easy separation of the two PCR products on denaturing polyacrylamide gels and for quantitation of the radioactive bands with a PhosphorImager (Fig. 6A). Using QC-PCR, we have clearly demonstrated that the intracellularly expressed RZs cleaved endogenous IGF-II mRNA.

Interestingly, in our study, single and double RZs showed almost the same level of efficacy in reduction of IGF-II mRNA in PC-3 cells. This observation can be explained in the fol-

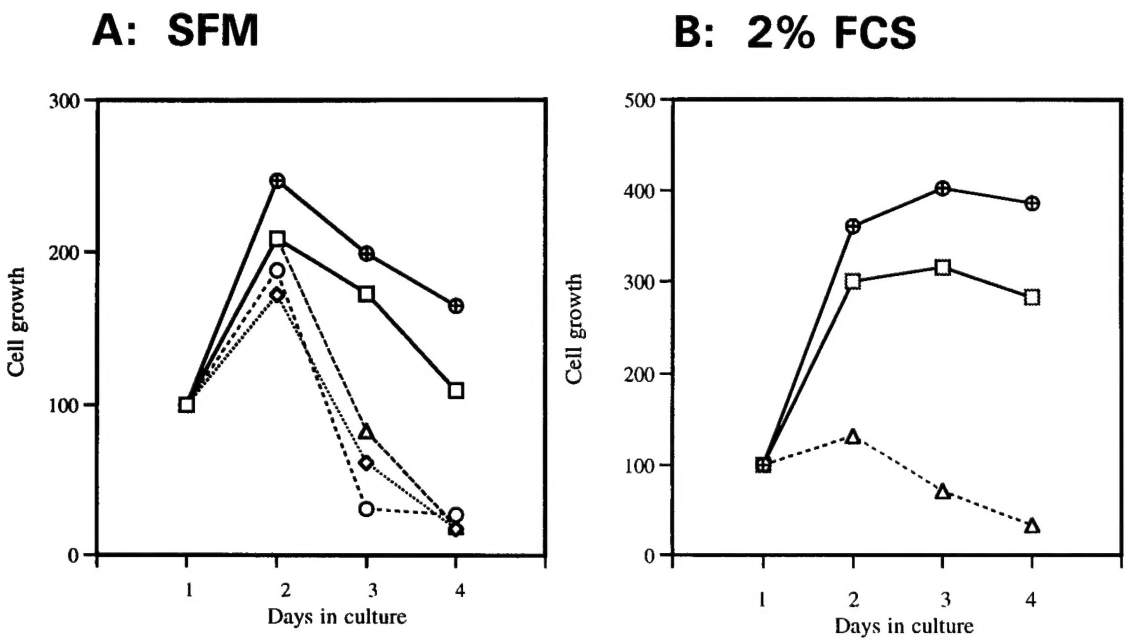


FIG. 8. Effect of RZ expression on PC-3 cell growth as measured by the MTT method. Cell growth under serum-free conditions (A) or in the presence of 2% FCS (B). Parental PC-3 cells (⊕), vector-control PC-3 cells (V37; □), R-expressing PC-3 cells (R4; ◇, R6; ○, and R39; △) were cultured either in serum-free medium (SFM) or in the presence of 2% FCS. Cell growth was determined for 4 days in triplicate by the MTT method. Growth curves are normalized by setting the OD value of day 1 as 100% for each transfectant. Shown are the representative results from three independent experiments.

TABLE 3. Effect of RZ expression on PC-3 cell growth as measured by time-lapse microscopy

	PC-3 cells	R-expressing transfectants			Vector transfectant V37
		R4	R6	R39	
Doubling time ± SEM (h)	28.3 ± 0.39	37 ± 1.1	38.6 ± 2.3	42.5 ± 2.8	32.3 ± 1.0
Doubling time (-fold) relative to parental PC-3 cells	1.00	1.31	1.36	1.50	1.14
		$P < 0.01$	$P < 0.001$	$P < 0.001$	$P > 0.05$

Cell division of parental PC-3 cells and transfectants in RPMI1640 in the presence of 10% FCS was recorded. Doubling time for each clone was calculated from five to eight divisions.

lowing ways: 1) due to unique secondary or tertiary structure caused by other cellular factors, interaction of IGF-II mRNA with the double RZ may make one of the cleavage domains unable to anneal with the substrate (second cleavage site); thus there is no cleavage reaction for that domain; or 2) the RZ-expressing clones selected may be only those that are able to moderately reduce IGF-II to a point that does not cause cell death.

Although *in vitro* studies of RZs are essential steps for quantitating their catalytic efficiencies, they cannot be used to predict the efficiency of the RZs when expressed in cells. Due to the complexity of eukaryotic gene expression, and also multiple factors involved, the RZ action in the cell is heavily influenced by the cellular environment. Factors that may interfere with RZ action in the cell include RZ expression level, colocalization of RZs with the target RNAs, location of the RZ gene relative to the target gene in the host genome, target sites at which the RZ functions, secondary structure of the target RNA or RZs, and cofactors or inhibitors such as proteins, Mg^{2+} concentration, and pH. For example, if RZ expression is not highly efficient, the level of RZs may be too low to cleave the target RNA. Even though RZs are efficiently expressed, if RZs are not in the same cell compartment with the target RNA, RZ activity would be dramatically decreased.

The secondary structure of the target RNA or RZs is also very important. Since RZs cleave the target RNA by first complementary binding to the target RNA, if the secondary structure of target RNA or RZs interferes with the binding of RZ and target, RZ activity will be reduced. Also, proteins may bind to portions of the RNA substrate or to the RZ itself and may facilitate or interfere with the catalysis. For example, previous studies demonstrated an enhancement of RZ cleavage reactions by RNA-binding proteins such as heterogeneous nuclear ribonucleoprotein A1 and the capsid protein NC7 of human immunodeficiency virus type 1 (HIV-1) (37, 45, 46). In our study, pTZU6 + 27 and pcDNA 3 vectors were used to express IGF-II RZs intracellularly under the control of RNA pol III and II, respectively. The RZ transcripts from pTZU6+27/RZ should be relatively stable and expressed in both nucleus and cytoplasm, but mainly in the nucleus (36, 47, 48). Pol III promoters, which naturally drive transfer RNA and small nuclear RNA synthesis, are good for high-level, non-tissue-specific expression of short RNAs. For example, Thompson *et al.* (41) used a transfer RNA-based RNA pol III promoter-driven RZ and found high accumulation of recom-

binant pol III RZ transcripts in all cell lines tested. The RZ activity was readily detectable in total RNA extracted from stably transformed human T cell lines. Michienzi *et al.* (48) used U1 small nuclear RNA chimeric RZs with substrate specificity for the Rev pre-mRNA of HIV and showed that this construct caused more efficient reduction in the Rev pre-mRNA level *in vivo*. Kawasaki *et al.* (49) reported that by using a pol III-driven vector, the adenoviral-E1A-associated 300-kDa protein expression in HeLa cells was inhibited. In contrast, the RZ transcripts from pcDNA3/RZ can be ubiquitously expressed in the cytoplasm under the control of RNA pol II. Pol II promoters, including viral promoters, which are used naturally for mRNA synthesis, can allow tissue-specific expression of RZs. A disadvantage of Pol II promoters is their requirement for long coding sequences, so that the RZ sequence may be placed in long RNA molecules. This can interfere with RZ conformation.

In the present study, both pol II and pol III promoter-driven vectors similarly expressed RZs and suppressed IGF-II mRNA levels in PC-3 cells, which resulted in cell growth inhibition. It should be noted that the growth of M-expressing PC-3 cells was not affected although the M RZ could have an antisense effect. Since the *in vitro* experiments revealed formation of a stable IGF-II RNA substrate/M RZ as seen in Fig. 2, formation of the complex in cells might also take place. Our results, however, indicate that if such a complex formed in PC-3 cells, it was not sufficiently stable to inhibit cell growth. It is possible that short RNA hybrids may be destroyed by ribosome translocation or heterogeneous nuclear ribonucleoproteins (37). The different results in the growth effects observed between R and M RZs thus support the notion that the catalytic activity of R RZ is required to significantly inhibit cell growth under our experimental conditions.

Suppression of IGF-II mRNA levels by the RZs was associated with reduced IGF-II protein levels. The reduction of protein levels was, however, not as significant as that of mRNA levels. This suggests that there may be a possible difference in the regulation of two IGF-II forms, the regular 7.5-kDa form and the incompletely processed 15-kDa form. The IGF-II protein levels measured in this study reflect those of both 7.5- and 15-kDa IGF-II since the RIA used an anti-IGF-II antibody that binds both forms of IGF-II. It is possible that the expression of the 15-kDa IGF-II, which is commonly expressed in cancerous tissues, is more affected by IGF-II RZ actions while the 7.5-kDa IGF-II level may not be affected. Further analysis of the expressed IGF-II protein by immunoblotting is necessary to substantiate this possibility. It is also necessary to examine whether expression of other IGF system components including IGF-binding proteins has been changed when an active RZ is intracellularly expressed.

RZ-expressing PC-3 cells did not grow well but rather die under serum-starved conditions while they grew in the medium supplemented by 10% FCS, which was the condition used for isolation of the RZ-expressing clones. It has been shown that PC-3 cells undergo apoptosis under serum-free conditions (50), and that IGFs are antiapoptotic (15–17). Consistent with those previous studies, the results shown in Fig. 8 show that PC-3 cell and vector-control cells seem to undergo apoptosis in SFM, and that the reduction of IGF-II levels by intracellular expression of IGF-II RZs seem to stimulate apoptosis of PC-3 cells in SFM and low serum medium. Investigation on mitogenic and apoptotic pathways of R-expressing PC-3 cells are now in progress.

In conclusion, the present study demonstrated that IGF-II RZs were active at least in one cell line and were able to lower endogenous IGF-II mRNA and protein levels, and that blockage of IGF-II/IGFIR signaling by IGF-II RZs inhibited cell growth. The IGF-II RZ can thus be used as a tool to investigate the role of IGF-II in other physiological systems. The study also provided the basis for the development of IGF-II RZs as future cancer therapeutics.

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